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A Novel Fully Validated LC-MS/MS Method for Quantification of Pyridoxal-5´-phosphate Concentrations in Samples of Human Whole Blood

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Abstract

Quantification of pyridoxal-5´-phosphate (PLP) in biological samples is challenging due to the presence of endogenous PLP in matrices used for preparation of calibrators and quality control samples (QCs). Hence, we have developed an LC-MS/MS method for accurate and precise measurement of the concentrations of PLP in samples (20 µL) of human whole blood that addresses this issue by using a surrogate matrix and minimizing the matrix effect. We used a surrogate matrix comprising 2% bovine serum albumin (BSA) in phosphate buffer saline (PBS) for making calibrators, QCs and the concentrations were adjusted to include the endogenous PLP concentrations in the surrogate matrix according to the method of standard addition. PLP was separated from the other components of the sample matrix using protein precipitation with trichloroacetic acid 10% w/v. After centrifugation, supernatant were injected directly into the LC-MS/MS system. Calibration curves were linear and recovery was > 92%. QCs were accurate, precise, stable for four freeze-thaw cycles, and following storage at room temperature for 17h or at -80 °C for 3 months. There was no significant matrix effect using 9 different individual human blood samples. Our novel LC-MS/MS method has satisfied all of the criteria specified in the 2012 EMEA guideline on bioanalytical method validation.

Introduction

Pyridoxal-5´-phosphate (PLP) is the biologically active form of vitamin B6. When dietary intake is low [1], its concentrations decline rapidly. PLP is a coenzyme in the catabolism of homocysteine and it has a critical role in the treatment of hyperhomocysteinemia [2]. PLP is also a cofactor in a large number of enzymatic reactions in the central nervous system (CNS) and its deficiency can cause neurological symptoms [3].

As PLP lacks a chromophore in its chemical structure, quantification using HPLC with ultraviolet detection is challenging due to poor assay sensitivity unless PLP is first derivatized. Methods involving derivatization of PLP with semicarbazide [4-6], cyanide [7-8],

chlorite [2] and bisulfite [9-12] have been reported. However, LC-MS/MS bioanalytical methods using stable isotope dilution techniques [1,3,13] are preferable because they have high sensitivity and selectivity, and time-consuming derivatization is usually unnecessary.

Quantification of PLP concentrations in biological samples such as human whole blood is challenging due to the presence of endogenous PLP in the sample matrix. Irrespective of which method of detection is used, a surrogate matrix for making calibration curves and quality control samples (QCs) is necessary. Most previous methods used water as matrix for making assay calibrators [2,8,9,11,14]. Other approaches include use of 4% albumin in PBS as matrix [13] or commercially supplied calibrators and QCs [4]. In other work [1], assay calibrators were made in the actual matrix (whole blood) and then corrected for the endogenous PLP concentration (ratio of intercept to slope from the calibration run) to estimate the PLP concentrations in 'unknown' samples. Whole blood samples from individuals with low and high endogenous PLP concentrations were also used as QCs. The main drawback of this latter approach is that concentrations of PLP in unknown samples that are lower than the endogenous concentration of PLP in the pooled whole blood matrix, will not be able to be quantified. Use of water as the surrogate matrix instead of human whole blood or plasma will result in a high matrix effect and inaccuracy in LC-MS/MS assay performance.

In healthy human subjects, PLP concentrations in plasma and red blood cells are highly correlated [6,15], whereas for critically ill patients, PLP concentrations are reportedly lower in plasma and higher in red blood cells [6,15]. This likely reflects the lower plasma albumin concentration in critically ill patients compared with healthy individuals, leading to an increase in PLP binding to red bloods cells in the critically ill patient population [15]. Hence, quantification of the PLP concentration in samples of whole blood is potentially a more robust indicator of vitamin B6 status in both healthy and critically ill individuals.

Herein, we describe a novel fully-validated high throughput LC-MS/MS bioanalytical method for the quantification of PLP in samples of human whole blood employing calibrators and QCs made in a surrogate matrix (2% BSA in PBS). The potential matrix effect was successfully circumvented by using small sample aliquots (20 µL). A second set of QCs was made in human whole blood and both sets of QCs were evaluated against calibration curves made using the surrogate matrix. Using this novel approach, we have established the first method to be fully validated to satisfy the requirements of the 2012 EMEA guideline on bioanalytical method validation [16]. Our method has now been applied to the quantification of PLP in samples of human whole blood collected in a clinical trial to assess the vitamin B6 status of ~600 clinical study participants.

Material & methods

Reagents and chemicals

PLP, BSA and PBS (PBS; 0.01M phosphate buffer, 0.0027M potassium chloride, 0.137M sodium chloride, pH=7.4 at 25 ºC), isopropyl alcohol and trichloroacetic acid were supplied by Sigma-Aldrich (Castle Hill, Sydney, Australia). Pyridoxal-5´-phosphate (methyl-D3) (PLP-d3) was from Buchem B.V. (Apeldoom, The Netherlands). HPLC grade (Lichrosolv®) methanol and acetonitrile were from Merck Millipore (Kilsyth, VIC, Australia). Formic acid was from Ajax Finechem purchased through Crown Scientific Pty Ltd (Sydney, NSW, Australia). Hydrochloric acid (32% w/v) was from Chem-Supply (Gillman, SA, Australia). Human whole blood samples (EDTA as anticoagulant) were purchased from BioCore Pty Ltd (Sydney, NSW, Australia).

Instrumentation and chromatographic conditions

Detection of PLP and PLP-d3 was achieved in ESI mode using a QTrap 5500 (AB Sciex, Concord, Ontario, Canada). The highest abundant product ions were selected for both analytes. The HPLC system comprised a Symbiosis Pharma System (SPARK Holland System (SPARK Hollans, Emmen, The Netherland) with a pair of binary LC pumps, a

Reliance autosampler (SPARK Holland, Emmen, The Netherland) and a Mistral column oven (SPARK Hollans, Emmen, The Netherland). A Symmetry® C18 column (2.1 mm × 100 mm, 3.5 µm; Waters, Sydney, NSW, Australia) and a Phenomenex Security Guard C18 column (Phenomenex, Sydney, Australia) were used for chromatographic separation of the analytes of interest from other matrix components. The autosampler and column oven temperatures were 10 °C and 35 °C, respectively and the sample injection volume was 10 µL. The instrument was controlled by Symbiosis Pro software for Analyst (V 2.1.0.0) in conjunction with the MS controlling software (Analyst software V1.6 from AB Sciex, Concord, Ontario, Canada).

The mobile phase comprised solvent A (0.2% formic acid in water) and solvent B (0.1% formic acid in methanol) and the mobile phase flow rate was 0.3 mL/min. The mobile phase gradient commenced at 10% solvent B for 1.5 min followed by a steady increase to 80% Solvent B at 3 min, which was maintained for 30 s. Next, the percentage of solvent B in the mobile phase was decreased to 10% over a 0.1 min period which was maintained until the end of the chromatographic run (5.0 min).

Preparation of stock solutions and QC samples

Stock solutions of PLP and PLP-d3 at 1 mg/mL were prepared in hydrochloric acid (0.1 M). Aliquots of the stock solutions (200 µL) were stored at -80 ºC. These were diluted serially using trichloroacetic acid (TCA 10% w/v) in water to generate standard solutions at 100 and 10 µg/mL (freshly prepared every day). Standard working solutions of PLP were prepared by diluting the standard solution at 10 µg/mL to 200, 160, 100, 50, 20, 10 and 5 ng/mL using a 10% TCA solution. The internal standard working solution was prepared by dilution of the standard solution of PLP-d3 at 100 µg/mL to 50 ng/mL using a 10% TCA solution.

A separate weighing of the PLP reference standard was used to make QCs. Two sets of QCs were prepared in two different matrices, viz 2% BSA in PBS and human whole blood.

Before preparation of QCs, the endogenous PLP concentration in whole blood was estimated using the method of standard additions. A range of PLP concentrations (0-200 ng/mL) were spiked into samples of human whole blood. The endogenous PLP concentration in unspiked samples of the same human whole blood used for method validation was estimated to be 22 ng/mL using linear regression (Analyst®, V1.6, AB Sciex, Concord, Ontario, Canada). The final concentrations of PLP in QCs prepared in 2% BSA in PBS were 5 ng/mL (QC LLOQ), 15 ng/mL (QCL), 100 ng/mL (QCM) and 150 ng/mL (QCH). Due to the high endogenous concentration of PLP in samples of human whole blood (22 ng/mL), the following three QCs were prepared, viz 28 ng/mL (QCL), 97 ng/mL (QCM) and 150 ng/mL (QCH) by spiking PLP at 6, 75 and 128 ng/mL respectively into samples of human whole blood (containing 22 ng/mL of endogenous PLP). Traces of PLP were present in the surrogate matrix (2% BSA in PBS) used for making QCs and calibrators. On each day of experimentation, the endogenous concentration of PLP in the 2% BSA in PBS surrogate matrix was estimated (-intercept/slope of the linear calibration curve). This value was added to the inverse predicted concentrations of PLP in unknown samples of human whole blood.

Sample preparation

Aliquots (20 µL) of patient whole blood samples or QCs were added to 1.5 mL microfuge tubes. While vortexing, 260 µL aliquots of a 10% aqueous solution of TCA were added to the samples drop wise, followed by 20 µL aliquots of the internal standard working solution (PLP-d3; 50 ng/mL). After vortexing for 5 min, tubes were left at room temperature for 30 min after which they were shaken vigorously for 5 min. Following a 1h incubation at room temperature, tubes were vortexed-mixed and centrifuged at 14000 g for 15 min to precipitate proteins and cellular debris. The supernatants were transferred to a 96-well plate and placed in the autosampler of the LC-MS/MS system. All sample preparation steps were conducted under reduced light conditions.

Method validation

Linearity

The concentration of endogenous PLP in the surrogate matrix (2% BSA in PBS) was determined by spiking 20 µL aliquots with PLP to achieve the following PLP concentrations, viz 5, 10, 20, 50, 100, 160 and 200 ng/mL. The endogenous PLP concentration was estimated using the method of standard additions (Analyst®, V1.6, AB Sciex). The concentrations of PLP in unknown samples of human whole blood and the human whole-blood QC samples were corrected for the endogenous concentration of PLP in the surrogate matrix.

Calibration curve linearity using 7 different PLP concentrations was assessed on six separate occasions as summarized in Table 1. The interpolated concentrations of the calibration standards, as well as the CV% of the six replicates had to be within ±15% of the nominal concentrations, except for the LLOQ where ±20% is acceptable.

Precision and accuracy

The accuracy and precision of the assay were assessed on 3 different days (between-run), and on the one day (within-run) using 6 replicates of high, medium, low and LLOQ QCs. Concentrations were quantified using a freshly prepared standard curve. Accuracy (mean percentage deviation from nominal concentrations in the replicate set), and precision (the CV% of the measured concentrations) ≤ ±15% were considered acceptable, except at the LLOQ which should not deviate by more than ±20%.

Matrix effect

The matrix factors (MF) for PLP and PLP-d3 were estimated using six different individual lots of human whole blood, by calculating the ratio of the peak area in the presence of matrix

(measured by analyzing blank matrix after extraction and then spiked with PLP), to the peak area in the absence of matrix (pure analyte solution) after correction for the endogenous concentrations of PLP in that matrix. The internal standard (IS) normalized MF was calculated by dividing the MF of PLP by the MF of PLP-d3. The CV% of the IS-normalized MF calculated from the 6 different lots of human whole blood should not be greater than 15%. The matrix effect was tested for two different spiked concentrations of PLP (6 and 128 ng/mL) in the six individual human whole blood matrices.

$$MF\,(PLP) = \frac{\text{Peak area of postspiked PLP} - \text{Peak area of endogenous PLP in the matrix}}{\text{Peak area of PLP in pure solutions}}$$

$$MF\,(IS) = \frac{\text{Peak area of postspiked IS}}{\text{Peak area of IS in pure solution}}$$

Recovery

The recoveries of PLP and PLP-d3 from both surrogate matrix (2% BSA in PBS) and pooled human whole blood were determined by dividing the area ratio of extracted (pre-spiked) to the un-extracted (post-spiked) samples for each of the low, medium and high QCs (n=3).

Carry-over

Analyte carry-over was assessed by injecting blank samples after the ULOQ sample. Due to the fact that PLP was present in 'blank' samples, the PLP peak area for the blank (injected before the standard curve) was deducted from the area of PLP in the blanks that were injected after the ULOQ.

$$Carryover = \frac{\text{Peak area of double blank injected after ULOQ} - \text{Peak area of double blank injected before Standard zero}}{\text{Peak area of the LLOQ} - \text{Peak area of double blank injected before Standard zero}} \text{X100}$$

Selectivity

Due to the presence of PLP in all blank matrices, the selectivity was assessed only for the internal standard. Six different lots of human whole blood (with and without spiking with internal standard) were individually analyzed. Interfering peaks should have a response less than 5% of that for the internal standard.

Dilution Integrity

To make a dilution QC, human whole blood was spiked with PLP at 1 µg/mL (giving a total concentration of 1022 ng/mL), and then diluted with 2% BSA in PBS (dilution factor=10). Precision and deviation from the nominal concentrations for 6 replicates after dilution should be ≤15%.

For calculation of the PLP concentration after sample dilution, the endogenous PLP concentration in the diluent matrix was taken into account.

$$Final\ concentration = \frac{(Conc\ of\ PLP\ in\ matrix \times 1) + (Endogenous\ Conc\ of\ PLP\ in\ diluent\ matrix \times 9)}{10}$$

The endogenous PLP concentration in the 2% BSA in PBS surrogate matrix was 4.5 ng/mL. After a 10-fold dilution, the expected concentration in 2% BSA in PBS was 106.25 ng/mL, as shown in the following equation.

$$Final\ concentration = \frac{(1022 \times 1) + (4.5 \times 9)}{10} = 106.25$$

Stability

Freeze and thaw stability

QC samples (low and high) made up in each of the 2% BSA in PBS surrogate matrix and human whole blood were subjected to 4 cycles of freeze and thaw, followed by analysis against a freshly prepared calibration curve.

Matrix short-term stability

QC samples (low and high) made up in each of the 2% BSA in PBS and human whole blood matrices were allowed to stand at room temperature for 5h and then analysed against a freshly prepared calibration curve.

Frozen Storage Stability

QC samples (low and high) made up in each of the 2% BSA in PBS surrogate matrix and human whole blood, and stored frozen at approximately -80°C were analyzed after 3 months and compared with freshly prepared QCs.

Stock solution stability

For stability tests, the percentage deviation from the nominal concentrations and the precision should not be > 15%. Stock solutions of each of PLP and PLP-d3 at 1 and 0.5 mg/mL, respectively, were prepared in 0.1M hydrochloric acid and stored at -80 °C. The stability of the stock solutions was tested after 49 days (PLP) and 42 days (PLP-d3) of storage at -80°C and after 17h at room temperature and protected from light.

Assay application

More than 600 samples of human whole blood collected from a clinical trial have been analyzed using this assay. Additionally, 10% of samples were re-analyzed to fulfil the Incurred Sample Reanalysis (ISR) requirement of the 2012 EMEA guideline on bioanalytical method validation. Concentrations determined upon initial analysis and after ISR should be within 20% of their mean for at least 67% of the repeat analyses [16].

Results and Discussion

MS and chromatography

For PLP and PLP-d3, positive ion electrospray ionization in MRM mode produced the best results. The MS parameters were optimized as follows: spray voltage 3000 V, turbo gas temperature 550°C, curtain gas pressure 35 psi, nebulizer gas pressure 60 psi, heater gas

pressure 60 psi and collision gas pressure set at medium. The collision energy was optimized at 23 V for PLP, and 25 V for PLP-d3.

For quantification, the transitions of the parent ions to the product ions were 248.0 →150.1 *m/z* for PLP and 251.0→153.1 for PLP-d3.

Acidic pH reduces the polarity of PLP promoting its retention on silica columns (log D= 1.8 at pH=2 vs logD= -2.8 at pH=7). In work by others [13], a very acidic mobile phase (650 mmol/L acetic acid and 100 mmol/L heptaflurobutyric acid in mobile phase) was used to retain PLP on a C8 column. Using a Waters Symmetry C18 column herein, facilitated use of a simple acidic mobile phase comprising two components (0.2% formic acid in water and 0.1% formic acid in methanol) in gradient mode (10% to 80% organic). PLP was eluted when the organic component of the mobile phase was approximately 50% at 2.6 min. A representative chromatogram showing the PLP and PLP-d3 peaks in a QC sample relative to a double-blank sample made in the 2% BSA in PBS surrogate matrix, is shown in Figure 1.

To prevent carryover, three successive needle washes were used as follows; A.0.2% formic acid in water, B. 40% acetonitrile, 40% methanol, 15% water, 5% isopropyl alcohol, C. 0.1% formic acid in 50% methanol in water.

Selection of sample preparation conditions

We used a 10% solution of TCA for precipitation of proteins in agreement with others [1,3,13]. As PLP is strongly protein bound, a 1h incubation was needed to liberate PLP from proteins [13]. Additionally, a small sample volume of only 20 µl was used and this contributed to the high extraction efficiency and low matrix effect.

Method validation

Linearity

A linear regression model with no weighting was applied to all calibration curves. A summary of the calibration curves prepared on 6 different days is shown in Table 1. The correlation coefficient (r^2) for the least squares line of best fit was > 0.99 for all 6 replicates. Additionally the precision (CV%) and accuracy (%) were ≤8.2% and 2.4%, respectively.

Precision and accuracy

The within-run and between-run precision (CV%) and accuracy (% deviation from the nominal concentrations) for 6 replicates of all QCs were ≤9.9% and 11.9%, respectively (Table 2).

Matrix effect

The matrix factor for the individual human whole blood samples spiked with PLP at 6 and 128 ng/mL and PLP-d3 at 50 ng/mL, are summarized in Table 3. The CV% of the matrix factor for human whole blood samples spiked with PLP at 6 and 128 ng/mL were ≤ 12% and 4.7%, respectively.

Recovery

The mean recovery of PLP from QCs prepared in each of the 2% BSA in PBS surrogate matrix and human whole blood were 93.9 and 92.0% respectively (Table 4). The mean recovery of PLP-d3 from QCs prepared in each of the 2% BSA in PBS surrogate matrix as well as pooled human whole blood were 96.9% and 89.7% respectively (Table 4). Using only one concentration of the analyte for testing recovery [1,13] can be misleading because the extraction method may behave differently in low and high concentrations and this is why we assessed both low and high concentrations of PLP .

Carry-over

No carry-over effect was observed across 3 days of experiments. The maximum carry-over was 3.4% of the peak area of the standard spiked with PLP at 5 ng/mL, which is

insignificant. No peak corresponding to the internal standard was observed in blanks that were injected after the ULOQ. Carry-over was also monitored during sample analysis and was found to be insignificant.

Selectivity

There were no peaks that interfered with the internal standard area in 6 different lots of human whole blood.

Dilution Integrity

Following dilution of a sample with a PLP concentration almost 5-fold higher than that of the ULOQ (1 µg/mL) with the 2% BSA in PBS surrogate matrix, the mean accuracy and precision were 3.1% and 2.8%, respectively.

Stability

Irrespective of whether QCs were prepared in the 2% BSA in PBS surrogate matrix or in human whole blood, they were stable for at least 4 cycles of freeze and thaw. Additionally, QCs were stable when stored for at least 6 h at room temperature and for at least 10 months of frozen storage at -80ºC (Table 5). After extraction of QCs and storage in the autosampler at 10ºC, samples were stable for at least 84 h.

Stock solutions of both PLP and PLP-d3 were stable at room temperature for at least 17 h. In addition, PLP and PLP-d3 stock solutions were stable when stored at -80ºC for at least 49 and 42 days, respectively. In work by others [1], stock solutions of PLP were stable for up to one year when stored frozen similarly.

Incurred sample reanalysis

Ten percent of human whole blood samples were reanalyzed and the results compared with the results from the initial sample analysis. PLP concentrations in 89% of the reanalyzed

samples were within 20% of the mean of the two analyses, thereby satisfying the ISR criterion in the 2012 EMA guideline on bioanalytical method validation [16].

Endogenous PLP Concentrations

Due to the lack of analyte-free matrix to prepare calibrators and QCs, it is difficult to compare results between laboratories [17]. In a recent comparison of an LC-ESI-MS/MS method for quantification of PLP in 250 µL aliquots of whole human blood with an HPLC method based on derivatization of PLP [1], a significant proportional bias of 11% was found in favor of the LC-ESI-MS/MS method. The main contributing factor was that the concentrations of PLP in the QCs did not take the endogenous PLP concentration in either the surrogate matrix or the human whole blood matrix into account, thereby introducing systematic errors. Hence, our methodological approach offers a novel and standardized approach for quantification of endogenous compounds in biological samples.

In our present work, we evaluated the impact on bioanalytical method performance of using 2% BSA in PBS as a surrogate matrix for making PLP calibrators and QCs, as the concentrations of plasma proteins and salts are not too dissimilar from those in human whole blood. Traces of PLP in the surrogate matrix (3-5 ng/mL) were quantified using the method of standard additions and the unknown samples were corrected for the endogenous PLP concentration in the 2% BSA in PBS surrogate matrix.

Using a small sample volume of only 20 µL in our PLP assay minimized dissimilarities between the surrogate matrix and samples of human whole blood. Using 2% BSA in PBS is preferable to water for making assay calibrators due to the high extent of PLP binding to serum albumin. To ensure that QCs made in surrogate matrix mimicked human whole blood samples in terms of assay performance, we prepared a second set of QCs by spiking PLP into samples of whole human blood, that were stored under the same conditions as study samples and QCs made using the surrogate matrix. The endogenous PLP concentration in human whole blood used to make the 2nd set of QCs was estimated using the method of

standard additions and the nominal concentrations of QCs were adjusted by adding the spiked PLP concentrations to the endogenous PLP concentration in that particular whole blood matrix. Both sets of QCs were analyzed during method validation and sample analysis.

In contrast to previous methods that used pooled human whole blood matrix with or without spiking with PLP [1,2,3,4,9,11,13], our bioanalytical method is the first to use QCs prepared by spiking PLP at LLOQ, Low, Med and High concentrations to cover most of the calibration range in accordance with the requirements of the 2012 EMEA guideline on bioanalytical method validation [16]. Apart from lack of knowledge about the integrity of human whole blood samples during storage, a major limitation associated with using pooled whole blood matrices for making calibrators and QCs for PLP assay, is lack of full coverage of the calibration range particularly at the lower end. These limitations also apply to commercially available QCs for use in PLP assays.

Using our novel approach for making the calibrators and QCs for the accurate and precise quantification of PLP in small samples (20 µL) of human whole blood, our bioanalytical method is the first that satisfies all of the requirements of the 2012 EMEA guideline on bioanalytical method validation [16]. Our method has now been successfully applied to the quantification of PLP in more than 600 samples of whole human blood collected from participants in a clinical trial, prior to and after a 6-month period of vitamin B6 supplementation. We have also successfully used the same approach for quantification of homocysteine, another endogenous compound in human serum, and the method has been validated successfully in accordance with the criteria specified in the 2012 EMEA guideline on Bioanalytical Method Validation [18].

Conclusion

Our novel approach employing 2% BSA in PBS as surrogate matrix in place of human whole blood (actual matrix) for making the PLP assay calibrators and QCs, together with a small

sample volume (20 µL), minimizes the matrix effect which underpins the excellent method performance demonstrated. More generally, our novel methodological approach has wide potential application to bioanalytical method development and validation for quantification of a broad range of endogenous vitamins and hormones in biological samples using LC-MS/MS.

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Table 1. Linearity of the PLP calibration standards

Standard Concentration (nominal, ng/mL)	**Measured concentrations (n=6)**	**Precision (CV%)**	**Accuracy (% deviation from nominal concentrations)**
5	4.8	8.2	-3.3
10	9.8	4.3	-2.4
20	19.7	2.4	-1.3
50	50.5	1.4	1.0
100	101	3.2	1.3
160	161	1.6	0.6
200	199	1.2	-0.6
Endogenous concentrations of PLP in the BSA 2% (mean)	4.2		
r^2 (mean)	0.9989		
Slope (mean)	0.02		

Table 2. Precision and accuracy of the QC samples prepared in a 2% BSA in PBS surrogate matrix as well as in pooled human whole blood

		Within Run 1 (n=6)		**Within Run 2 (n=6)**		**Within Run**
		Accuracy (% deviation from nominal concentrations)	Precision (CV%)	Accuracy (% deviation from nominal concentrations)	Precision (CV%)	Accuracy (% deviation from nominal concentrations)
QCs in 2% BSA	**LLOQ**	**-1.9**	**5.0**	**-10.5**	**15.1**	**1.2**
	Low	**0.1**	**1.9**	**-2.4**	**6.8**	**0.7**
	Med	**4.3**	**5.3**	**3.5**	**1.8**	**2.8**
	High	**-1.4**	**1.3**	**1.4**	**1.9**	**1.8**
QCs in Human Whole blood	**Low**	**11.9**	**4.3**	**9.8**	**4.2**	**-1.7**
	Med	**-0.9**	**2.8**	**3.4**	**2.0**	**0.4**
	High	**1.8**	**1.3**	**2.0**	**3.6**	**0.9**

Blank Matrix ID	PLP Matrix Factor (6 ng/mL)	Internal Standard Matrix Factor (50 ng/mL)	IS Normalized MF	PLP Matrix Factor (128 ng/mL)	Internal Standard Matrix Facto (50 ng/mL
1	0.36	0.41	0.86	0.35	0.36
2	0.27	0.35	0.78	0.30	0.31
3	0.30	0.35	0.85	0.28	0.29
4	0.33	0.35	0.94	0.30	0.32
5	0.26	0.34	0.75	0.29	0.34
6	0.19	0.28	0.67	0.27	0.29
	Mean		0.81		
	CV%		11.85		

Table 3. Matrix effect in six individual lots of human whole blood

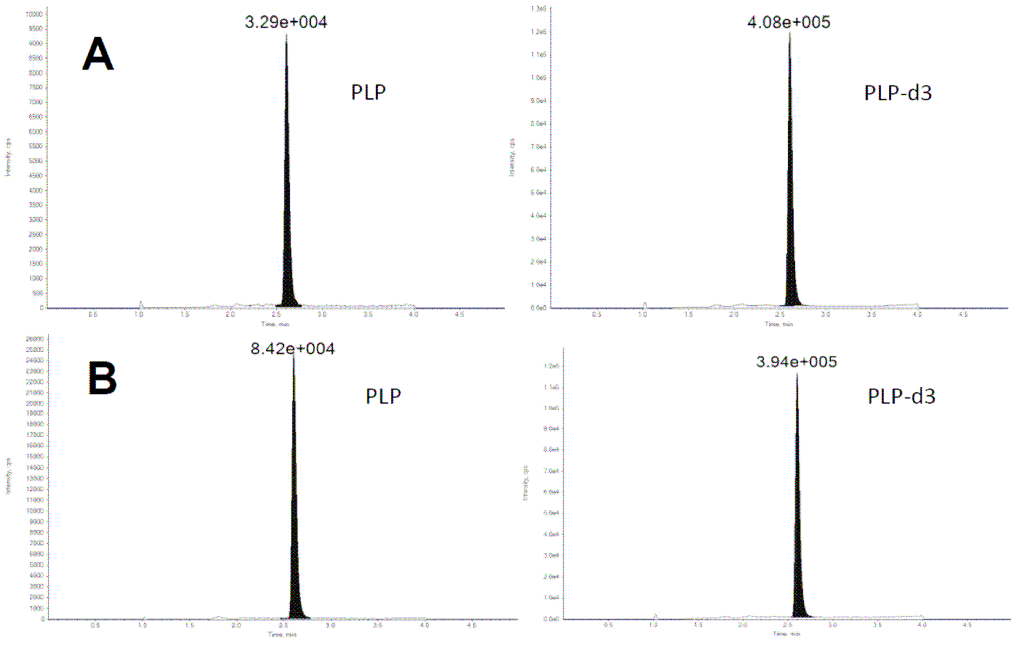
Table 4. Recovery of PLP from 2% BSA in PBS surrogate matrix compared with pooled human whole blood

		Recovery PLP (n=3)	Recovery IS (n=3)
2% BSA	Low	95.1	96.9
	Med	93.6	
	High	94.7	
Mean		93.9	
Pooled human whole blood	Low	95.9	89.7
	Med	89.8	
	High	89.7	
Mean		92.0	

Table 5. Stability of the PLP QC samples

F & T Stability (4 Cycles)								Room temperature stability (6 h)								Frozen	
Whole blood				2% BSA				Whole blood				2% BSA				Whole	
Accuracy (% deviation from nominal concentration)		Precision (CV%)		Accuracy (% deviation from nominal concentration)		Precision (CV%)		Accuracy (% deviation from nominal concentration)		Precision (CV%)		Accuracy (% deviation from nominal concentration)		Precision (CV%)		Accuracy (% deviation from nominal concentration)	
QCL	QCH	QCL	QCH	QCL	QCH	QCL	QCH	QCL	QCH	QCL	QCH	QCL	QCH	QCL	QCH	QCL	QCH
-2.8	0.7	4.0	4.1	2.8	1.3	1.9	1.6	-2.6	-3.0	2.5	4.9	3.1	1.9	2.0	1.8	-0.1	1.2

Fig 1. Representative chromatograms of PLP and PLP-d3 spiked into samples of human whole blood; A. St 0, B. LLOQ (5 ng/mL)



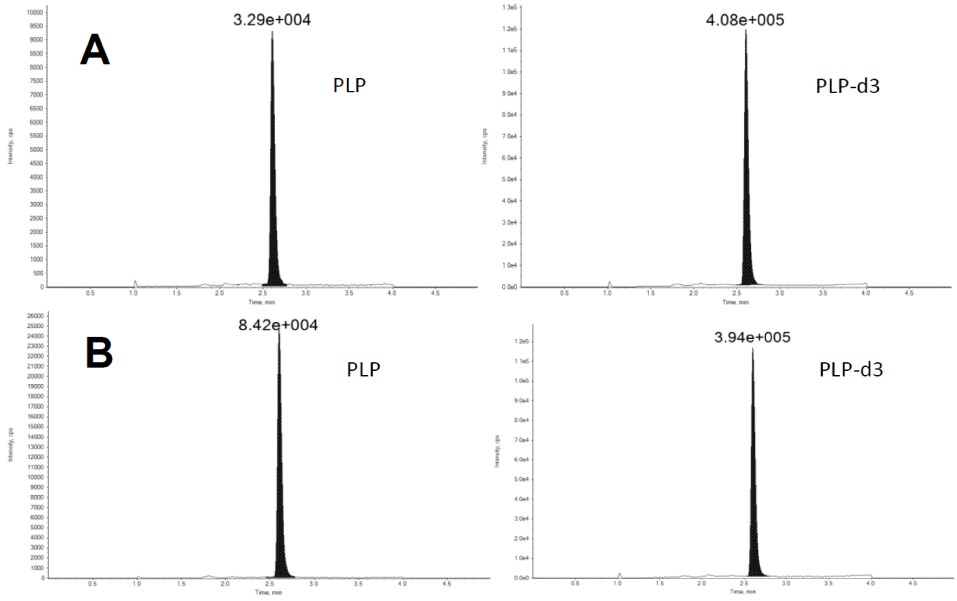


Figure 1 .